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Construction of Rubella Virus Genome-Length cDNA Clones and Synthesis of Infectious RNA Transcripts

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Plasmids containing a complete cDNA copy of the rubella virus (RUB) genomic RNA were constructed. Transfection into cell culture of genome-length RNA transcribed in vitro from one of these cDNA clones, Robo102, resulted in the production of virus which preserved the genetic and phenotypic characteristics of the parental virus from which the cDNA clone was derived. Prior to construction of the RUB genome-length cDNA clones, the 5′-terminal sequence of the RUB genomic RNA was determined to be 5′CAAUGG. . .3′ following the cap structure. Analysis of the specific infectivity of RUB genomic RNA isolated from virions revealed that in Vero cells, the specific infectivity of RUB genomic RNA is roughly equivalent to that of Sindbis virus genomic RNA. In RUB virion RNA preparations, the subgenomic RNA was detected. It was demonstrated that subgenomic RNA was packaged into RUB virions; however, the presence of the subgenomic RNA was not essential for infectivity of the genomic RNA.

Rubella virus (RUB), the etiological agent of German measles (rubella), is the sole member of the *Rubivirus* genus in the *Togaviridae* family (18). The genome of RUB is a single-stranded RNA which is capped at the 5′ terminus and polyadenylated at the 3′ terminus (29). In RUB virions, the genomic RNA is encapsidated in a quasi-spherical nucleocapsid composed of multiple copies of a virus-specified capsid protein, C, which in turn is surrounded by a lipid bilayer envelope in which two virus-specified glycoproteins, E1 and E2, are embedded (16, 28, 44). Hovi and Vaheri (17) and Sedwick and Sokol (39) reported that the genomic RNA is infectious (and thus of positive polarity). In both of these reports, it was noted that the infectivity of the RUB genomic RNA was relatively low.

The nucleotide sequence of the RUB genomic RNA has been determined except for the identity of the two or three nucleotides at the 5′ terminus (7, 10, 11). The length of the 3′-terminal poly(A) tract has also not been established. Open reading frame (ORF) analysis revealed that there are two long ORFs contained in the RUB genomic RNA, a 5′-proximal ORF that encodes nonstructural proteins and a 3′-proximal ORF (SP-ORF) that encodes the three viral structural proteins in the order NH_2-C-E2-E1-COOH. In RUB-infected cells, a subgenomic RNA which encompasses the 3′-terminal 3,327 nucleotides of the genomic RNA serves as the mRNA for the translation of the SP-ORF (12, 29). The subgenomic RNA species has been detected in virion RNA preparations (27, 29, 39). The other virus-specific RNA species is a negative-polarity RNA of genome length which is detected in RNA extracted from RUB-infected cells and is present only in double-stranded RNA complexes (15). The only known function of the negative-polarity RNA is to serve as a template for the synthesis of the two positive-polarity RNA species. Both the genomic organization and RNA replication strategy of RUB are similar to those of the alphaviruses, members of the other togavirus genus.

The study of RUB has progressed slowly because of the slow replication rate and low virus yield in infected cell culture. Additionally, panels of temperature-sensitive mutants, which have been extremely useful in the study of the molecular biology of other RNA viruses, have not been developed with RUB. Genome-length cDNA clones constructed for the synthesis of infectious genome-length RNA transcripts in vitro by using SP6 or T7 RNA polymerase have allowed rapid and extensive analysis of the molecular biology of positive-polarity RNA viruses of both plants (8, 19, 31, 43) and animals (21, 32, 42). Such genome-length cDNA clones have been developed for several alphaviruses, including Sindbis virus (SIN), Venezuelan equine encephalitis virus, Ross River virus, and Semliki Forest virus (5, 20, 23, 33). The SIN and Semliki Forest virus genome-length cDNA clones were further developed into broad-host-range vectors for high-level expression of foreign proteins in eukaryotic cells (2–4, 47). In this report, successful construction of a genome-length cDNA clone of RUB from which infectious RNA can be transcribed will be described.

MATERIALS AND METHODS

Cells and viruses. Vero cells and BHK-21 cells were grown at 35°C under 5% CO_2 in Dulbecco's modified Eagle medium (Gibco/BRL) containing gentamicin (50 μg/ml) and supplemented with 5% fetal bovine serum for Vero cells or 7.5% fetal bovine serum–10% tryptose phosphate broth for BHK-21 cells. Two variants of the Therien strain of RUB were used in this study. One, obtained from J. Wolinsky and designated W-Therien, forms turbid plaques on Vero cells following 6 days of incubation. It was from this variant that cDNA clones were generated for determining the genome sequence (7) and constructing the infectious clone. The second variant, obtained from J. Chantler and designated F-Therien, was selected in the laboratory of Ralf Petterson for rapid release. This variant produces clear plaques on Vero cells within 5 days. The F-Therien variant was plaque purified twice in Vero cells and then amplified at a low multiplicity of infection in Vero cells to

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TABLE 1. Full-length cDNA clones of RUB

RNA polymerase promoter	5′ terminus of RNA transcript[a]	Vector (infectivity of RNA transcript)[b]		
		pGEM2	pUC18	pCL1921
SP6	GCA-UGGAAG	Robo1 (−)		
SP6	GCAAUGGAAG	Robo2 (+→−)	Robo12 (+→−)	Robo102 (+)
T7	GCAAUGGAAG	Robo3 (−)	Robo13 (−)	
T7	GGCAAUGGAAG	Robo4 (−)	Robo14 (−)	

[a] Genome sequence is CAAUGGAAG.
[b] +, infectious; −, noninfectious; +→−, infectious when first generated but gradually losing infectivity during passage and amplification in *E. coli*.

produce infecting stocks of virus used in this study. SIN (HR strain), obtained from J. Strauss, was plaque purified six times in BHK-21 cells and amplified at a low multiplicity of infection in BHK-21 cells to produce infecting stocks.

RNA manipulations. For virion purification, culture fluid was harvested from RUB-infected Vero cells on days 3 through 10 postinfection and from SIN-infected BHK-21 cells at 2 days postinfection, and virions were purified by sequential velocity and isopycnic density gradient centrifugation as described previously (37). The virus band in the isopycnic density gradient was collected and stored at 4°C until use without pelleting of the virus. Virion RNA was prepared by mixing the isopycnic density gradient-purified virus suspension with 2 volumes of LET buffer (5% dodecyl lithium sulfate, 10 mM Tris [pH 7.4 to 7.8], 2 mM EDTA) containing 400 μg of proteinase K (Boehringer Mannheim) per ml followed by phenol-chloroform extraction and ethanol precipitation in the presence of 20 μg of glycogen per ml as a carrier. In RNase protection assays, the purified virus suspension was incubated in the absence or presence of RNase A (final concentration of 100 ng/ml) for 30 min at 37°C. Following addition of RNasin (Promega) to a final concentration of 1 U/μl, virion RNA was extracted as described above. Intracellular RNA was extracted from infected cell cultures by the method of Sawicki et al. (38). Analysis of RNA samples by Northern (RNA) hybridization was done as described previously (15) except that a nylon transfer membrane (MSI) was used instead of nitrocellulose paper. The concentration of genomic RNA in virion RNA specimens was determined by Northern hybridization in comparison with dilutions of in vitro genome-length RNA transcripts of known concentration as standards. *Eco*RI-linearized Robo12 (Table 1) and *Xho*I-linearized Toto1101 (obtained from C. Rice) were used as templates for the transcription of genome-length RNA standards. The Northern blots were probed with a ^{32}P-labeled negative-polarity RNA which corresponds to 3′-terminal 587 nucleotides of the RUB genome (15) or ^{32}P-labeled DNA nicked translated from Toto1101 (for RUB and SIN RNAs, respectively). Dideoxy sequencing of RNA transcribed in vitro or extracted from RUB-infected cells was performed essentially as described in the "Promega Protocols and Applications Guide," with minor modifications. The negative-sense primer used had the sequence 5′TTTCTCCAT GGGAATGG3′ (oligonucleotide 43), which is complementary to nucleotides 33 to 49 of the genome.

Primer extension and determination of the primer extension product sequence. Primer extension using poly(A)$^+$ RNA from RUB-infected cells as a template and a ^{32}P-labeled, negative-sense primer with the sequence 5′TGGTCTCTTACCCAA CT3′ (oligonucleotide 36), which is complementary to nucleotides 101 to 117 of the genome, was carried out as previously described (12). The primer extension product (PEP) was purified by electrophoresis on an 8% polyacrylamide–urea sequencing gel (36) and 3′-terminally tailed with dATP, using terminal deoxynucleotidyltransferase (TdT; Boehringer Mannheim). The poly(A)-tailed PEP was amplified by PCR, using oligonucleotide 36 and a positive-sense primer, 5′ACGTG CATGCCTGCAGT$_{20}$3′ (oligonucleotide 51), which consists of an anchor sequence made up of *Sph*I and *Pst*I restriction sites 5′ to an oligo(dT) tail. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM deoxynucleoside triphosphates, 25 pmol of each oligonucleotide, and 1 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) in a 100-μl volume. The amplification protocol consisted of 5 cycles of 1 min at 55°C and 3 min at 45°C followed by 25 cycles of 1 min at 94°C, 1.5 min at 55°C, and 2.5 min at 72°C. The amplified products were digested with *Nco*I (at nucleotide 40 of the genome) and *Pst*I and ligated into the multiple cloning site of pGEM-5Zf(+) vector (Promega). Plasmids containing the PEP insert were sequenced by the dideoxy method, using the M13 forward sequencing primer. The PCR-amplified PEP was also subjected to asymmetric amplification using 5′-^{32}P-labeled oligonucleotide 43 as a primer. The amplification protocol was 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C for 40 cycles. The amplification products were purified from a sequencing gel and sequenced by the Maxam and Gilbert chemical sequencing method (26).

RNA 5′-3′ ligation and sequencing. The cap structure of RUB virion RNA was removed by digestion with tobacco acid pyrophosphatase (TAP; Epicentre Technologies) prior to the ligation of the 5′ and 3′ termini of the RNA, using T4 RNA ligase (New England Biolabs) (24). The ligated RNA was subjected to primer extension using oligonucleotide 36 as a primer as described above with the exception that Superscript murine leukemia virus reverse transcriptase (Gibco/BRL) was used instead of avian myeloblastosis virus reverse transcriptase. Subsequently, PCR amplification was performed with oligonucleotide 36 and a positive-sense primer with the sequence 5′AATGCCCGAGTGGATCCA3′ (oligonucleotide 52) which is colinear with nucleotides 9158 to 9175 of the genome. In this PCR, Hot-Tub DNA polymerase (Amersham) was used essentially as recommended by the manufacturer. The PCR protocol consisted of 1 min at 94°C, 2 min from 94 to 47°C, 2 min from 47 to 72°C, and 2 min at 72°C for 30 cycles. The amplified products were digested with *Nco*I (at nucleotide 40 of the genome) and *Apa*I (at nucleotide 9705 of the genome) and ligated into the multiple cloning site of pGEM-5Zf(+). Following identification and amplification of recombinant plasmids, dideoxy sequencing of the insert was performed with the SP6 RNA polymerase promoter primer (Promega).

RNA transfection and plaque assay. Lipofectin-mediated RNA transfection was done by the method of Rice et al. (32) except that Opti-MEM I reduced-serum medium was used instead of phosphate-buffered saline. Vero cells were incubated with the transfection mixture for 20 min, and BHK-21 cells were incubated for 10 min. Following transfection, Vero

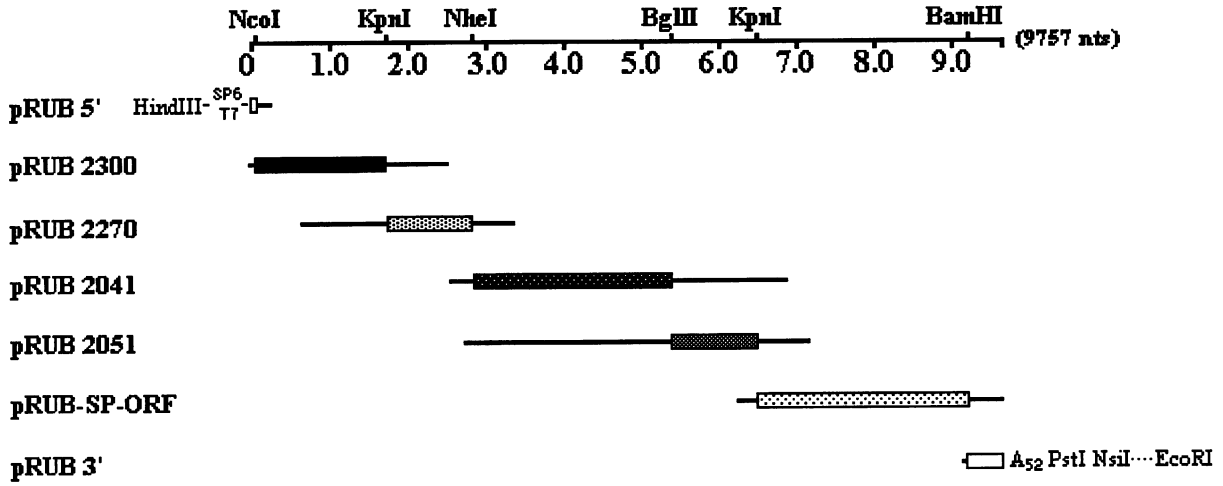

FIG. 1. Strategy for construction of the RUB genome-length cDNA clone. Numbers in the RUB genome scale refer to distance from the 5′ end in kilobases. pRUB 2300, pRUB 2270, pRUB 2041, pRUB 2051, and pRUB-SP-ORF are clones used in sequence determination (7, 10, 11, 25). pRUB 5′ and pRUB 3′ were generated by PCR amplification of cDNA transcribed from RUB virion RNA with the desired modifications included in the PCR primers. nts, nucleotides.

cells and BHK-21 cells transfected with SIN RNA were washed once with Opti-MEM I and overlaid with plaque assay agar (0.4% Oxoid no. 1 in Eagle's minimal essential medium containing 1% fetal bovine serum, 0.1% DEAE-dextran, and 50 μg of gentamicin per ml). Monolayers of BHK-21 cells do not remain viable long enough for the 5 to 6 days required for RUB to produce visible plaques. Therefore, to assay the specific infectivity of RUB RNA in BHK-21 cells, transfected cells were allowed to recover in BHK-21 growth medium for 2 h and were then trypsinized. Aliquots of the trypsinized, transfected BHK-21 cells were mixed with 3×10^6 freshly trypsinized Vero cells, and the suspension was added to 60-mm-diameter plates containing 4 ml of Vero growth medium. When most of the cells had attached (3 to 4 h), the medium was removed and the cells were overlaid with plaque assay agar. Plaques were visualized by removing the agar overlay and staining the cells with 0.1% crystal violet in formalin.

Construction of genome-length RUB cDNA clones. cDNA clones derived for determination of the nucleotide sequence of the RUB genome (7) were ligated together at convenient restriction sites as shown in Fig. 1. Specific modifications were used to produce both the 5′ and 3′ termini. For the 5′ terminus (pRUB 5′), to place the promoter for phage SP6 or T7 RNA polymerase immediately upstream of the RUB 5′ sequence, primer extension on RUB virion RNA using oligonucleotide 36 was followed by PCR amplification using oligonucleotide 36 and a second oligonucleotide which contained, in order from 5′ to 3′, a *Hin*dIII site, the SP6 or T7 promoter sequence, and a stretch of 17 to 20 nucleotides starting from nucleotide 1 of the RUB genome. In these PCRs, *Taq* DNA polymerase (Perkin-Elmer Cetus) was used essentially as recommended by the manufacturer. Four different synthetic oligonucleotides were used to generate various pRUB 5′ clones shown in Table 1: 5′TTCGAAGCTTATTTAGGTGACACTATAGCATGGAAGCTATCGGACCTC3′ for Robo1, 5′TCGAAGCTTATTTAGGTGACACTATAGCAATGGAAGCTATCGGA3′ for Robo2, 5′TCGAAGCTTAATACGACTCACTATAGCAATGGAAGCTATCGGA3′ for Robo3, and 5′TCGAAGCTTAATACGACTCACTATAGGCAATGGAAGCTATCGGA3′ for Robo4. The amplification products were restricted with *Hin*dIII and *Nco*I (at nucleotide 40 of the genome) and introduced into a plasmid which contained the 5′-terminal 2.5 to 2.6 kb of the genome (pRUB 2300). The insert between the *Hin*dIII and *Sna*BI sites (at nucleotide 1208 of the genome) in the new pRUB 2300 recombinant was excised and transferred into the full-length clone. For the 3′ terminus (pRUB 3′), a poly(A) tract was added to the full-length clone to replace the oligo(A)-oligo(G) stretch present in pRUB-SP-ORF (25). Oligonucleotide 51 was used to prime the reverse transcription reaction on RUB virion RNA, and then PCR amplification using *Taq* DNA polymerase in the presence of oligonucleotide 52 was performed. The product was inserted into the *Bam*HI (at nucleotide 9170 of the genome) and *Pst*I sites of an M13 mp18 vector. The recombinant M13 containing pRUB 3′ with the longest poly(A) tract (52 A residues) was transferred into pRUB-SP-ORF. The insert between two *Pst*I sites in pRUB-SP-ORF was excised and transferred into the genome-length clone. The completed genome-length clone was linearized at the *Nsi*I site which was immediately downstream of the poly(A) tract and *Pst*I site. The final genome-length clone contains a *Hin*dIII site at the 5′ terminus (upstream from the RNA polymerase promoter) and an *Eco*RI site at the 3′ terminus (downstream from the *Nsi*I linearization site) which can be used to excise the construct for transfer among various plasmid vectors.

In vitro transcription. *Nsi*I-linearized genome-length cDNA clones were used as templates for in vitro runoff RNA transcription. Since *Nsi*I digestion generates a 3′ overhanging end, the DNA templates were treated with Klenow DNA polymerase prior to the transcription reaction. 5′ capped transcripts (for infectivity assays) were synthesized at 37°C for 1 h in 25-μl reaction mixtures containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1 U of RNasin per μl, 1 mM each ATP, CTP, GTP, and UTP, 2 mM cap analog [m^7G(5′)ppp(5′)G], 1.25 μg of linearized DNA template, and 25 to 40 U of SP6 or T7 RNA polymerase. RNAs lacking cap structures (for RNA sequencing) were synthesized by eliminating the cap analog in the reaction mixtures. The transcription reaction mixtures were used directly for cell transfection and RNA sequencing without

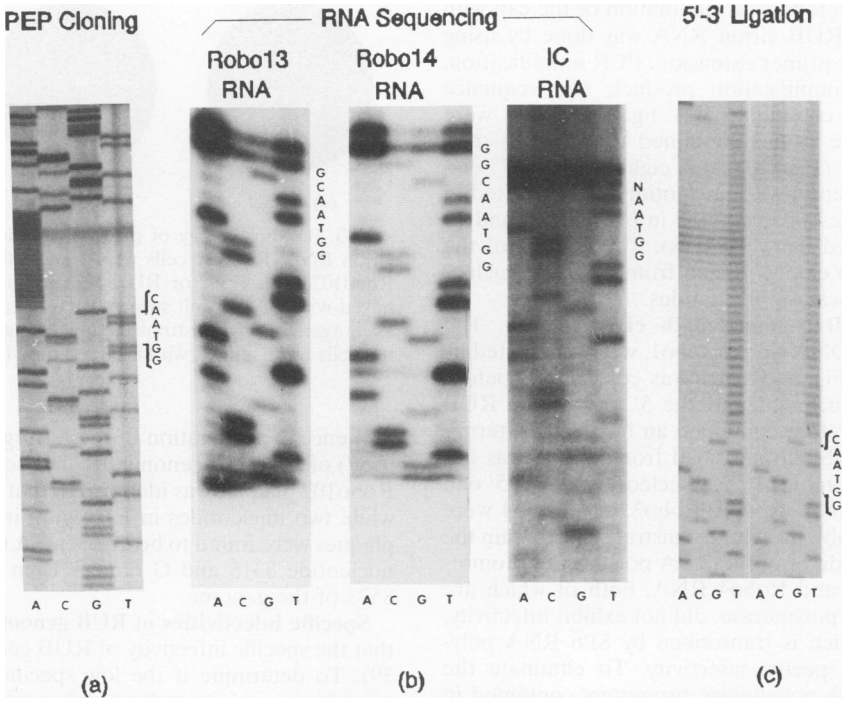

FIG. 2. 5′-terminal sequence of the RUB genomic RNA determined by different approaches. The sequence of the 5′-terminal six nucleotides as determined by each method is shown at the right of each gel. In each reaction, the sequencing primer was complementary to the RUB RNA, and thus the sequences given are complementary to the nucleotides in the lanes at the bottom of the gel. (a) Determination of PEP sequence. The PEP obtained by using poly(A)$^+$ intracellular RNA as a template and oligonucleotide 36 as a primer was purified and subjected to 3′ dA tailing, PCR amplification, DNA cloning, and dideoxy sequencing. Sequencing of one of two clones recovered that contained the PEP is shown. (b) Dideoxy sequencing of RNA. RNA templates were uncapped RNA transcripts of Robo13 or Robo14 (Table 1) or RNA extracted from RUB-infected Vero cells (IC RNA). The primer used was oligonucleotide 43. The known sequence of the 5′ end of the Robo13 and Robo14 transcripts is given. The sequence given by the IC sequencing ladder is based on the finding that the top band of the Robo13 and Robo14 sequencing ladder does not correspond to a nucleotide in the transcripts. (c) Sequence of 5′-3′-ligated RUB genomic RNA. TAP-decapped RUB virion RNA was subjected to 5′-3′ ligation, primer extension, PCR amplification, DNA cloning, and dideoxy sequencing. The sequence ladders of two clones (out of eight sequenced) containing the complete 5′-3′ junction are shown.

purification of the RNA transcripts by phenol-chloroform extraction.

RESULTS

Determination of the nucleotides at the 5′ end of the RUB genomic RNA. For construction of a RUB genome-length cDNA clone, it was necessary to determine the identity of the nucleotides at the 5′ end of the RUB genomic RNA. In the communication in which the sequence of the RUB genomic RNA was reported (7), dideoxy sequencing using poly(A)$^+$ RNA extracted from RUB-infected cells as a template and oligonucleotide 36 (complementary to nucleotides 101 to 117 of the genome) as a primer resulted in a sequencing ladder with readable sequence of 5′ATGG. . .3′ below a wide dark band in all four lanes. From the width of the band, it was concluded that there were most likely two unidentified nucleotides at the 5′ terminus, and thus the sequence was reported as 5′NNAUGG. . .3′. As shown in Fig. 2b, dideoxy sequencing using RNA extracted from RUB-infected cells as a template and oligonucleotide 43 (complementary to nucleotides 33 to 49 of the genome) as a primer resulted in a sequencing ladder topped with two dark bands across all four lanes and an extra resolvable nucleotide, A, under these dark bands. The presence of two dark bands is commonly encountered in dideoxy sequencing using capped RNA as a template (1, 14). Recent experiments have shown that the bottom band corresponds to the 5′-terminal nucleotide and the top band is an artifact (6, 40). As shown in Fig. 2b, dideoxy sequencing of uncapped in vitro transcripts from Robo constructs with known sequence showed that this phenomenon also occurs with uncapped RNAs and verified that in our hands, the bottom band corresponded to the authentic 5′ nucleotide. Thus, the sequence at the 5′ terminus of the RUB genome is 5′NAA UGG. . .3′. It has been shown that the 5′ nucleotide can be resolved despite the presence of dark bands in all four lanes by treatment of the sequencing reaction products with TdT to remove non-dideoxy-terminated products (6). However, treatment of the sequencing reaction from RNA from RUB-infected cells by TdT eliminated both bands completely (data not shown).

To determine the identity of the 5′ nucleotide, primer extension was done with poly(A)$^+$ RNA from RUB-infected cells as a template and ^{32}P-labeled oligonucleotide 36 as a primer, and the resulting PEP was isolated and subjected to 3′-terminal poly(A) tailing with TdT, PCR amplification, and cloning of the amplification product. Two clones containing the PEP were recovered, and both had the sequence 5′CA ATGG. . .3′ downstream from the T tract, which is complementary to the A tract added to the PEP by TdT (Fig. 2a). The chemically determined sequence of the product of asymmetric amplification of the initial PCR product obtained by using ^{32}P-labeled oligonucleotide 36 was also 5′(T_n)CAATGG. . .3′ (data not shown).

In a second approach, following elimination of the cap with TAP, 5′-3′ ligation of RUB virion RNA was done by using RNA ligase followed by primer extension, PCR amplification, cloning of the PCR amplification product, and sequence analysis. Sixteen clones containing 5′-3′ ligation inserts were sequenced. Eight of the clones contained the poly(A) tract followed by CAATGG (Fig. 2c), and eight had the 5′ end deleted by 3 to 12 nucleotides (3 nucleotides were deleted in four clones, 9 nucleotides were deleted in two clones, and 12 nucleotides were deleted in two clones). The lengths of the poly(A) tracts in the 16 clones ranged from 36 to 85 nucleotides; the mean length was 53 nucleotides.

Construction of RUB genome-length cDNA clones. The initial genome-length cDNA clone, Robo1, was constructed by the strategy shown in Fig. 1. Robo1 was constructed before determination of the nucleotides at the 5′ end of the RUB genomic RNA and therefore contained an incorrect 5′ terminus. Not surprisingly, RNA transcribed from Robo1 was not infectious. After determination of the nucleotides at the 5′ end of the RUB genomic RNA, Robo2, Robo3, and Robo4 were derived from Robo1 (Table 1). These constructs all contain the correct 5′ terminus but differ in the RNA polymerase promoters used. Robo3 RNA and Robo4 RNA, both of which are transcribed by T7 RNA polymerase, did not exhibit infectivity, while Robo2 RNA, which is transcribed by SP6 RNA polymerase, exhibited low specific infectivity. To eliminate the native SP6 and T7 RNA polymerase promoters contained in the pGEM2 vector used in construction of Robo2, Robo3, and Robo4, the RUB genomic cDNA inserts in Robo2, Robo3, and Robo4 were excised and transferred into pUC18. The new clones were named Robo12, Robo13, and Robo14. Robo13 and Robo14 RNA transcripts were not infectious; however, Robo12 RNA transcripts exhibited low specific infectivity. Disappointingly, the infectivity of Robo12 RNA transcripts was inconsistent and was eventually lost following passage of the plasmid. Recently, it was observed that maintenance of genome-length cDNA clones in high-copy-number plasmids leads to accumulation of mutations or deletions which results in loss of infectivity of transcripts synthesized from these clones (21, 32, 41). Therefore, the Robo2 RUB genomic cDNA insert was transferred to the low-copy-number plasmid pCL1921 (22), and the Sure strain of *Escherichia coli* (*uvrC umuC sbcC recB*, deficient in homologous recombination; Stratagene) was used as the host. The new clone was named Robo102. RNA transcribed from Robo102 was infectious, and infectivity was retained when the plasmid was maintained in Sure cells. The specific infectivity was roughly 5 to 10 plaques per 10 μg of RNA transcripts in BHK-21 cells.

The cDNAs used to assemble the Robo genomic cDNA clones were derived from the W-Therien variant of RUB. The W-Therien variant produces turbid plaques in Vero cells that generally require 6 days to develop. In the time since construction of the Robo clones was initiated, the F-Therien variant of RUB has replaced W-Therien as the standard virus used in the laboratory. The F-Therien variant is more cytopathic than the W-Therien variant; e.g., the F-Therien variant produces clear plaques in Vero cells within 5 days. As can be seen in Fig. 3, the plaques produced by Robo102 RNA transcripts retained the turbid plaque morphology phenotype of its W-Therien parent and were distinguishable from the clear plaques produced by the F-Therien variant genomic RNA. To further exclude the possibility of contamination of Robo102 RNA by virions or genomic RNA from the F-Therien variant, viruses eluted from individual plaques produced by F-Therien virion RNA and Robo102 RNA transcripts were used to infect Vero cells, and RNA extracted from the infected cells was subjected to sequence determination of the E1 region (nucleotides 8253 to 9757) of the RUB genome (9). The sequence of virus from the Robo102 plaques was identical to that in the Robo102 plasmid, while two nucleotides in E1 region in virus from virion RNA plaques were found to be different: A (versus G in Robo102) at nucleotide 8315 and G (versus U in Robo102) at nucleotide 8523 of the genome.

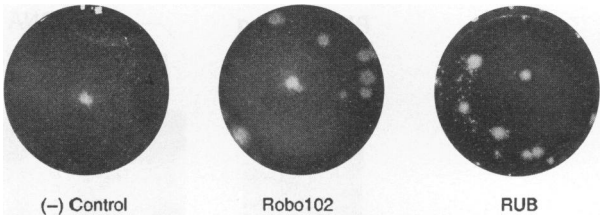

FIG. 3. Morphology of plaques produced by Robo102 RNA and virion RNA. BHK-21 cells were transfected with H_2O [(−) control], Robo102 transcripts, or RUB (F-Therien) virion RNA, trypsinized, mixed with a Vero cell suspension, plated, and overlaid with plaque assay agar. Six days posttransfection, the agar overlay was removed and the cells were stained with 0.1% crystal violet in 10% formalin.

Specific infectivities of RUB genomic RNA. It was reported that the specific infectivity of RUB genomic RNA was low (17, 39). To determine if the low specific infectivity of Robo102 transcripts was in part due to inherent low specific infectivity of RUB genomic RNA, the specific infectivities of RUB and SIN genomic RNAs isolated from virions were determined. Virions were purified by velocity and isopycnic density centrifugation; genomic RNA was extracted and analyzed by agarose gel electrophoresis and Northern hybridization. In Fig. 4a is a Northern blot of RUB virion RNA and intracellular RNA from RUB-infected cells probed with ^{32}P-labeled RNA with sequence complementary to the 3′-terminal 587 nucleotides of

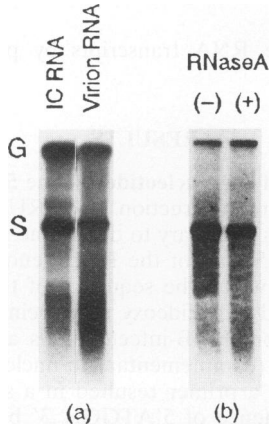

FIG. 4. Analysis of virus-specific RNA species present in highly purified RUB virion preparations. RNA samples were denatured with dimethyl sulfoxide and glyoxal, electrophoresed on 1% agarose gels, and transferred to a nylon membrane. The nylon membrane transfers were hybridized against a ^{32}P-labeled negative-sense RNA probe complementary to the 3′-terminal 587 nucleotides of the RUB genome. (a) RNA extracted from RUB-infected Vero cells (IC RNA; left lane) and a RUB virion preparation (right lane). On the left margin, the positions of genomic (G) and subgenomic (S) RNAs are indicated. (b) RNase protection assay. Aliquots of a RUB virion preparation were incubated in the absence (left lane) or the presence (right lane) of 100 ng of RNase A per ml, and then RNA was extracted from virions.

the RUB genome. In addition to the genomic RNA, a smaller RNA species that comigrated with the subgenomic RNA was present in the virion RNA preparations. No subgenomic RNA was present in preparations of SIN virion RNA (data not shown). Subsequently, a Northern blot similar to that in Fig. 4a was probed with two ^{32}P-labeled oligonucleotides with sequences complementary to stretches of 20 nucleotides either upstream (oligonucleotide 11) or downstream (oligonucleotide 10) from the 5′ end of the subgenomic RNA (12). Oligonucleotide 11 hybridized exclusively with RUB genomic RNA, while oligonucleotide 10 reacted with both RNA species (data not shown), indicating that the smaller RNA species present in virion RNA preparations was the subgenomic RNA. Both the genomic and subgenomic RNAs in virion RNA preparations were sensitive to digestion with RNase A (data not shown), and thus neither was present in double-stranded form. Consistent with this observation, negative-polarity RNA was not detectable in Northern hybridization blots of virion RNA preparations probed with a positive-polarity RUB-specific RNA probe (data not shown). To determine whether the subgenomic RNA is packaged or adheres to the virion surface, an RNase protection assay was performed. As shown in Fig. 4b, both the genomic and subgenomic RNAs were protected, demonstrating that the subgenomic RNA is packaged in RUB virions.

To investigate whether the subgenomic RNA is essential for infectivity, virion RNA was denatured in 90% dimethyl sulfoxide (to eliminate potential genomic-subgenomic RNA complexes) and recovered by ethanol precipitation. A series of twofold serial dilutions was made and used to transfect cells. As shown in Fig. 5, the relationship between plaque number and RNA dilution was linear, indicating that this is a first-order reaction and that the subgenomic RNA is not essential for infectivity.

The amount of genomic RNA in SIN and RUB virion RNA preparations was quantitated, and the RNA preparations were used in Lipofectin-mediated cell transfections. The specific infectivities are given in Table 2. The specific infectivity of RUB genomic RNA was equivalent in Vero and BHK-21 cells; however, the specific infectivity of SIN genomic RNA was fivefold higher in BHK-21 cells than in Vero cells. In Vero cells, the specific infectivity of SIN genomic RNA was twice that of RUB genomic RNA, while in BHK-21 cells, SIN genomic RNA was 10-fold more infectious than RUB genomic RNA.

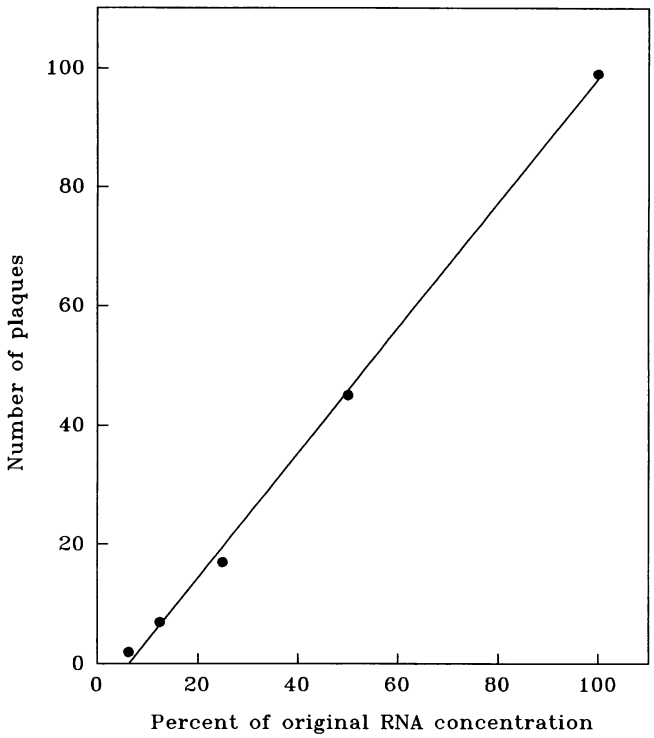

FIG. 5. Relationship between plaque number and virion RNA dilution. A RUB virion RNA preparation was denatured in 90% dimethyl sulfoxide at 50°C for 5 min and recovered by ethanol precipitation. The precipitated RNA was dissolved, and a series of twofold dilutions was made and used to transfect duplicate plates of Vero cells. The line is derived by linear regression.

DISCUSSION

In this report, we described the construction of a genomic cDNA clone of RUB from which infectious RNA transcripts could be synthesized, a development which should be useful in the study of the molecular biology of RUB. The specific infectivity of RNA transcribed from Robo102, the current infectious clone, is 5 logs lower than that of viral genomic RNA. RNA transcripts from infectious clones of other viruses are generally 1 to 2 logs less infectious than viral genomic RNA (20, 33, 34, 42). The reason for the low specific infectivity of Robo102 transcripts is not known. With the infectious clones of other viruses, the presence of authentic sequence at both the 5′ and 3′ termini is important for infectivity (8, 32, 42). Because of the specificity of both SP6 and T7 RNA polymerases, the addition of at least one extra G at the 5′ end of the Robo RNA transcripts is unavoidable. The presence of the G may adversely affect the infectivity. Also, at the 3′ end of Robo102 RNA transcripts are seven nonviral nucleotides downstream from poly(A) tract. We are in the process of removing these additional 3′ nucleotides. We are also generating new cDNA clones for restriction fragment replacement in Robo102 in case unrecognized deleterious mutations exist in the Robo102 sequence (33). Finally, we determined the specific infectivities of F-Therien and W-Therien genomic RNAs and found them to be similar (data not shown). Thus, the low infectivity of Robo transcripts is not due to inherent low specific infectivity of the W-Therien variant genomic RNA from which the Robo clones were derived.

It was reported that the infectivity of RUB genomic RNA was low (17, 39). However, no standard of comparison was given in either of these reports. We originally thought that the low specific infectivity of the Robo transcripts was due to the inherent low specific infectivity of the RUB genomic RNA. However, we found that the specific infectivity of RUB genomic RNA was only 2- to 10-fold lower than that of SIN genomic RNA, depending on the cell line transfected, and thus the low specific infectivity of the Robo transcripts was due to

TABLE 2. Specific infectivity of RUB and SIN genomic RNAs

Virus	Specific infectivity (PFU/μg of genomic RNA)[a]	
	Vero	BHK-21
RUB	9.3×10^4	9.7×10^4
SIN	1.9×10^5	1.0×10^6

[a] Each value is the average of at least two experiments each with two independent preparations of RUB virion and one preparation of SIN virion RNA.

factors other than low specific infectivity of the RUB genomic RNA. We found the specific infectivity of SIN genomic RNA to be 10^6 PFU/μg in BHK-21 cells, using Lipofectin-mediated transfection. This is higher than the specific infectivities reported for SIN, Semliki Forest, and Ross River virus genomic RNAs in BHK-21 cells with use of DEAE-dextran-mediated transfection, which range from 1.5×10^4 to 5×10^5 PFU/μg (20, 23, 33), but lower than the specific infectivity of Semliki Forest virus RNA of 6.8×10^6 PFU/μg achievable by electroporation (23). The specific infectivity of SIN genomic RNA was fivefold higher in BHK-21 cells than in Vero cells, a finding that presumably reflects greater efficiency of transfection in BHK-21 than in Vero cells. In contrast, the specific infectivity of RUB genomic RNA was equal in BHK-21 and Vero cells. This relatively lower specific infectivity of RUB genomic RNA in BHK-21 cells could have been due to the extra step used in transfection of BHK-21 cells with RUB genomic RNA (trypsinization, cell attachment). Also, it was reported that a higher percentage of cells in Vero cell cultures were initially infectable with RUB in comparison with BHK-21 cells in that Hemphill et al. (15) found that 46 to 47% of the Vero cells gave rise to infectious centers early in infection when the multiplicity of infection exceeded 5 PFU per cell, while Wong et al. (46) and Sedwick and Sokol (39) found that only 3 to 10% of the BHK-21 cells gave rise to infectious centers under similar conditions. If the difference was due to the intracellular factors, the same factors could also reduce the specific infectivity of RUB genomic RNA in BHK-21 cells.

In the course of development of the RUB infectious clones, two other features of the RUB RNA species were analyzed. First, the nucleotides at the 5′ and 3′ ends of the RUB genomic RNA were characterized. The nucleotide at the 5′ end of the RUB genomic RNA was identified as a C, an unusual finding since the 5′ nucleotide of the genomic RNAs of other capped positive-polarity RNA viruses has been found to be a purine (30). The mean length of the 3′-terminal poly(A) tracts in the clones generated by 5′-3′ ligation and PCR amplification was 53 nucleotides. This was probably an underestimate due to the presence of RNase in commercially available TAP as documented by other researchers (24) and apparent in the finding that half of the clones that we isolated contained 5′-terminal deletions (these deletions were only of from 3 to 12 nucleotides). We know of no other study in which the length of the poly(A) tract was characterized by this method. In comparison, the poly(A) tract on alphavirus RNAs ranges from 45 to 200 nucleotides, with a mean length of 68 nucleotides (13).

Second, we showed that the presence of subgenomic RNA in RUB virion RNA preparations, a finding noted in other reports (27, 29, 39), was due to packaging of the subgenomic RNA into virion particles. The presence of the subgenomic RNA in virion RNA preparations was not essential for infectivity of the genomic RNA. Whether the genomic RNA and subgenomic RNA are packaged into the same virion particle or into different particles remains to be resolved. Since it is possible that these species are packaged into the same particle, perhaps as a complex or an aggregate, it cannot at this point be concluded that the subgenomic RNA contains a packaging signal. In most alphaviruses, the subgenomic RNA is not packaged (we found that RNA extracted from SIN virions purified identically to RUB virions contained no subgenomic RNA). The packaging signal of SIN has been located between nucleotides 745 and 1225 of the genomic RNA (45). Interestingly, it was recently reported that Aura virus packages subgenomic RNA into virions (35). The Aura virus genomic and subgenomic RNA are packaged into separate virion particles (40a).

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